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Michael Jewett

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14. ABSTRACT
We will develop cell-free technologies for endowing the translation apparatus (the ribosome and its peripheral machinery) with new chemical capabilities. The ribosome is the cell's factory for protein synthesis, stitching together natural amino acid substrates into sequence-defined polymers (proteins) from a defined template. Expanding the repertoire of ribosome substrates and functions promises novel drugs that kill bacteria resistant to common antibiotics and might enable new classes of materials having tunable properties such as shape memory and self-healing. Such an expansion has been a difficult task, however, because cell viability severely constrains the

15. SUBJECT TERMS
synthetic ribosomes, cell-free protein synthesis, ribosome purification, ribosome assembly, genome engineering, in vitro compartmentalization, emulsions

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Michael Jewett
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Report Title

Final Report: Engineering Synthetic Ribosomes (Topic 9.1)

ABSTRACT

We will develop cell-free technologies for endowing the translation apparatus (the ribosome and its peripheral machinery) with new chemical capabilities. The ribosome is the cell's factory for protein synthesis, stitching together natural amino acid substrates into sequence-defined polymers (proteins) from a defined template. Expanding the repertoire of ribosome substrates and functions promises novel drugs that kill bacteria resistant to common antibiotics and might enable new classes of materials having tunable properties such as shape memory and self-healing. Such an expansion has been a difficult task, however, because cell viability severely constrains the changes that can be made. To overcome this limitation, we will combine in vitro ribosome synthesis and self-assembly with ribosome display to select and evolve synthetic ribosomes that are programmed to synthesize non-natural polymers of defined sequence. Our work will play a transformative role in efforts to understand sequence-function design rules that bring new chemistry to biology. Moreover, it could open the way to new classes of chemicals and materials.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received		Paper
01/16/2017	16	Filippo Caschera, Jin Woo Lee, Kenneth K. Y. Ho, Allen P. Liu, Michael C. Jewett. Cell-free compartmentalized protein synthesis inside double emulsion templated liposomes with in vitro synthesized and assembled ribosomes, Chem. Commun., ( ): 5467. doi:
TOTAL:	**1**	

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received	Paper
TOTAL:	

Number of Papers published in non peer-reviewed journals:

(c) Presentations

- Jewett, M.C. Repurposing ribosomes. Bioorganic Chemistry Gordon Research Conference, Andover, NH, June 2016
-Jewett, M.C. Repurposing ribosomes. Xenobiology Conference XB2, Berlin, Germany, May 2016.
-Jewett, M.C. Repurposing the translation apparatus for synthetic biology. Naval Research Laboratory, Washington DC, May 2016.
-Jewett, M.C. Repurposing the translation apparatus for synthetic biology. Department of Chemistry, University of Toronto (Invited by Students). Toronto, Canada, May 2016.
-Jewett, M.C. Synthetic Biology: Life redesigned. North Atlantic Treaty Organization (NATO) Strategic Foresight Analysis Workshop, Luzern, Switzerland, April 2016.
-Jewett, M.C. Repurposing the translation apparatus for synthetic biology. Department of Energy, Environmental & Chemical Engineering, Washington University, St. Louis, April 2016.
-Jewett, M.C. Repurposing the translation apparatus for synthetic biology. Department ofChemical and Biological Engineering, Tufts University, Medford, MA, February 2016.
-Jewett, M.C. Repurposing ribosomes for synthetic biology. Department of Chemical Engineering, Stanford University. Stanford, CA. February 2016.
-Jewett, M.C. Repurposing the translation apparatus for synthetic biology. Structural & Quantitative Biology Seminar. Departments of Molecular and Cellular Biology and Chemistry, UC Berkeley. Berkeley, CA. January 2016.
-Jewett, M.C. Repurposing ribosomes for synthetic biology. Department of Chemical and Biomolecular Engineering, University of Wisconsin. Madison, WI, December 2015.
-Jewett, M.C. Cell-free synthetic biology. Gen9, Boston, MA. December 2015.
-Jewett, M.C. Cell-free synthetic biology. National Academy of Sciences & The Royal Society. Forum on Synthetic Biology and Gain of Function, and Implications for Regulation. Chicheley, Buckinghamshire, UK. November 2015
-Jewett, M.C. Establishing cell-free synthetic biology for the production of therapeutics, materials and energy. Department of Chemical and Biomolecular Engineering, Rice University. Houston, TX. October 2015.
-Jewett, M.C. A new cell-free framework for pathway prototyping and enzyme discovery. Genomatica, San Diego, CA October 2015.
- Jewett, M.C. Establishing designer translation systems for synthetic biology. Department of Biomedical Engineering. University of California, Irvine, CA. October 2015.

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

01/16/2017 14.00 Ashty S Karim, Quentin M Dudley, Michael C Jewett. Cell?Free Synthetic Systems for Metabolic Engineering and Biosynthetic Pathway Prototyping, : Wiley VCH Verlag GmbH & Co. KGaA, (2017)

01/16/2017 15.00 Jessica G Perez, Jessica C Stark, Michael C Jewett. Cell-Free Synthetic Biology: Engineering Beyond the Cell, : Cold Spring Harbor Lab, (2017)

TOTAL: **2**

Patents Submitted

1. Jewett, M.C., Hong, S.H., Kwon, Y.C., Martin, R.W., and Des Soye, B.J. 2014. Methods for improved in vitro protein synthesis with proteins containing non standard amino acids. U.S. Patent Application Serial No.: 62/044,221.

2. Jewett, M.C. and Fritz, B.R. 2014. Method to In Vitro Ribosome Synthesis and Evolution. U.S. Patent Application Serial No.: 62/098,622.

3. Jewett, M.C., Des Soye, B. 2016. A Highly Productive One-Pot System for the Incorporation of Non-Standard Amino Acids into Cell-Free Synthesized Proteins. U.S. Patent Application Serial No. 62/362,988

Patents Awarded

Awards

- American Chemical Society Biological Technologies Division Young Investigator Award
- Suzanne C and Duncan A Mellichamp Distinguished Lecturer, Department of Chemical and Biomolecular Engineering, Georgia Institute of Technology
- Camille Dreyfus Teacher-Scholar Award
- Engineering Biology Research Consortium, Inaugural Academic Research Member
- Invited Speaker and Synthetic Biology Technical Expert, North Atlantic Treaty Organization (NATO) Strategic Foresight Analysis Workshop
- Conference Chair, Synthetic Biology, Engineering, Evolution, and Design (SEED)
- Invited Speaker, National Academy of Sciences & The Royal Society - Forum on Synthetic Biology and Gain of Function, and Implications for Regulation

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Yi Liu	1.00	
FTE Equivalent:	**1.00**	
Total Number:	**1**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Filippo Caschera	1.00
FTE Equivalent:	**1.00**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Michael Jewett	0.10	
FTE Equivalent:	**0.10**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 5.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 5.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 2.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 4.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME	
Yi Liu	
Total Number:	**1**

Names of other research staff

NAME	PERCENT_SUPPORTED
Lauren Clark	0.20
FTE Equivalent:	**0.20**
Total Number:	**1**

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attachment.

Technology Transfer

The work in this proposal led to a newly funded MURI award - W911NF-16-1-0372. Engineering the translation apparatus for synthesis of electronically active sequence-defined polymers.

As a result, we are starting to interact with Jim Sumner from ARL, among others.

Engineering Synthetic Ribosomes

Final Progress Report 2015-2016
ARO Biochemistry Program
W911NF-11-1-0445

Michael C. Jewett, Northwestern University

Final Progress Report
In the final year of the project, we have made significant progress, including several publications (one at *Chemical Communications*) and 15 oral presentations. In addition, we submitted one new provisional patent application. In addition, we successfully competed for follow-on funding with a MURI proposal. I also received the ACS Biochemical Technologies Young Investigator Award, chaired the 2016 Synthetic Biology, Evolution, Engineering, and Design meeting, and spoke at NATO on synthetic biology.

Stated project goals
The ultimate goal of this research is to engineer synthetic ribosomes in order to synthesize unique materials with atomic-scale resolution over architecture, functionality, and reactivity. Novel function is endowed by incorporating non-standard amino acids (nsAAs) into protein biopolymers. To achieve this vision, we need:

- tRNAs that can be amino acylated with unnatural amino acids (**Aims 1 & 2**)
- New codons that can be assigned to new amino acids (**Aims 1 & 2**)
- Ribosomes that allow for the formation of new polymers (**Aims 3 & 4**)

In the past period, we have focused on (i) encapsulating the *in vitro* ribosome construction platform in emulsion droplets and (ii) testing variant ribosomes with active site point mutations that cannot be studied in cells for their ability to synthesize proteins.

The highlights of the year are listed below:

- Developed a cell-free expression platform for making bacterial ribosomes encapsulated within giant liposomes capable of synthesizing sfGFP.
    - The liposomes were prepared using a double emulsion template, and compartmentalized *in vitro* protein synthesis was analyzed using spinning disk confocal microscopy.
- Generated tens of plasmids encoding mutant ribosomes.
- Developed and adapted our cell-free ribosome construction platform to rapidly assess the activity and fidelity of mutant ribosomes.
- Invited Speaker and Synthetic Biology Technical Expert, North Atlantic Treaty Organization (NATO) Strategic Foresight Analysis Workshop
- Conference Chair, Synthetic Biology, Engineering, Evolution, and Design (SEED)
- Invited Speaker, National Academy of Sciences & The Royal Society - Forum on Synthetic Biology and Gain of Function, and Implications for Regulation

- Delivered 15 lectures, including invited seminars on engineering ribosomes at the Bioorganic Chemistry Gordon Research Conference, the Xenobiology conference (XB2), and a Keynote Lecture at the European Society of Biochemical Engineering Sciences.
- Named a Camille Dreyfus Teacher Scholar
- Awarded the ACS Biochemical Technologies Young Investigator Award

Current and future work

- Submit a manuscript on improving cell-free protein synthesis through genome engineering of *Escherichia coli* lacking release factor 1
- Submit a manuscript on ribosome display for ribosome evolution
- Submit a manuscript on using in vitro synthesized ribosomes to produce proteins with site-specifically introduced unnatural amino acids.

Issues

None

Awards and special honors

2017	American Chemical Society Biological Technologies Division Young Investigator Award
2016	Suzanne C and Duncan A Mellichamp Distinguished Lecturer, Department of Chemical and Biomolecular Engineering, Georgia Institute of Technology
2015	Camille Dreyfus Teacher-Scholar Award
2016	Engineering Biology Research Consortium, Inaugural Academic Research Member
2016	Invited Speaker and Synthetic Biology Technical Expert, North Atlantic Treaty Organization (NATO) Strategic Foresight Analysis Workshop
2016	Conference Chair, Synthetic Biology, Engineering, Evolution, and Design (SEED)
2015	Invited Speaker, National Academy of Sciences & The Royal Society - Forum on Synthetic Biology and Gain of Function, and Implications for Regulation

Leadership and professional service

2016-present	Mentor, Mentored Discussions of Teaching, Center for the Integration of Teaching, Research, and Learning, Northwestern University, Evanston, IL
2016	CAPS program of the Lurie Cancer Center internal grant review committee
2016-present	Member, Innovation and New Ventures Office (INVO) Faculty Committee, Northwestern University, Evanston, IL
2016	Scientific Review Board, Chicago Biomedical Consortium Catalyst Program
2015-present	Founding co-director, Northwestern Center for Synthetic Biology, Northwestern University, Evanston, IL
2015-2016	Member, Molecular Biosciences Faculty Search Committee, Northwestern University, Evanston, IL
2015-present	Member, Office for Research Limited Submissions Advisory Committee, Northwestern University, Evanston, IL
2015-2016	CLP Strategic Planning Committee, Northwestern University, Evanston, IL

2015-present Biotechnology Training Program Steering Committee, Northwestern University, Evanston, IL

Service to professional societies

2016-2019 Division 15 Chair, Food, Pharmaceutical, and Bioengineering Division, AIChE
2016 Conference Chair, Synthetic Biology, Engineering, Evolution, and Design (SEED), Chicago, IL
2016 Division 15 Vice Chair, Food, Pharmaceutical, and Bioengineering Division, AIChE
2016 Chair, Microbial Process Development, 251st ACS National Meeting, San Diego, CA
2016 Conference Chair, 6th International Conference on Biomolecular Engineering, Singapore
2016 Area Programming Chair, Division 15c, Bioengineering: Food, Pharmaceutical, and Bioengineering Division, AIChE National Meeting, San Francisco, CA
2015-present Member, US/EU biotechnology task force synthetic biology work group
2015 Member, Synthetic Biology Standards Consortium

Service to government agencies

2016-present Consultant, cell-free systems thrust, Applied Research for the Advancement of S&T Priorities (ARAP) in Synthetic Biology for Military Environments (SBME)
2016 Invited Speaker and Synthetic Biology Technical Expert, North Atlantic Treaty Organization (NATO) Strategic Foresight Analysis Workshop, Luzern, Switzerland, April 19-20.

Patent applications

- Jewett, M.C., Hong, S.H., Kwon, Y.C., Martin, R.W., and Des Soye, B.J. 2014. Methods for improved *in vitro* protein synthesis with proteins containing non standard amino acids. U.S. Patent Application Serial No.: 62/044,221.
- Jewett, M.C. and Fritz, B.R. 2014. Method to In Vitro Ribosome Synthesis and Evolution. U.S. Patent Application Serial No.: 62/098,622.
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Publications

- Caschera, F.,† Lee, J.W.,† Ho, K. K. Y., Liu, A.P.,* Jewett, M.C.* 2016. Cell-free compartmentalized protein synthesis inside double emulsion templated liposomes with *in vitro* synthesized and assembled ribosomes. *Chemical Communications.* 52: 5467-5469.
- Des Soye, B., Patel, J., Isaacs, F.J., * and Jewett, M.C.* 2015. Repurposing the translation apparatus for synthetic biology. *Current Opinion in Chemical Biology, In press.*
- Fritz, B.R., Jamil, O.K.%, and Jewett, M.C.* 2015. Implications of macromolecular crowding and reducing conditions for *in vitro* ribosome construction. *Nucleic Acids Research. In press – online.*
- Kwon, Y.C. and Jewett, M.C.* 2015. High-throughput preparation methods of crude extract for robust cell-free protein synthesis**.** *Scientific Reports.* 5:8663.

- Hong, S.H.,[†] Kwon, Y.C.,[†] Martin, R.W., Des Soye, B.J., de Paz, A.M., Swonger, K.N.[%], Ntai, I., Kelleher, N.L., and Jewett, M.C. * 2015. Improving Cell-free Protein Synthesis through Genome Engineering of *Escherichia coli* lacking Release Factor 1. *ChemBioChem.* 16:698-698. *(On the inside cover)*
- Hong, S.H., Kwon, Y.C., and Jewett, M.C. 2014. Non-standard amino acid incorporation into proteins using *Escherichia coli* cell-free protein synthesis. *Frontiers in Chemistry. 10 June 2014* | *doi: 10.3389/fchem.2014.00034*
- Palmer, M.J. and Jewett, M.C. 2014. Enabling a next generation of synthetic biology community organization and leadership. *ACS Synthetic Biology.* 3:117–120.
- Hong, S.H., Ntai, I., Haimovich, A.D., Kelleher, N.L., Isaacs, F.J., and Jewett, M.C. 2014. Cell-free protein synthesis from a release factor 1 deficient Escherichia coli activates efficient and multiple site-specific non-standard amino acid incorporation. *ACS Synthetic Biology.* 3: 398–409.
- Jewett, M.C.* and Patolsky, F.* 2013. Nanobiotechnology: synthetic biology meets materials science. *Current Opinion in Biotechnology.* 24:551-554.
- Jewett, M.C., Fritz, B.R., Timmerman, L.E., and Church, G.M. 2013. In vitro integration of ribosomal RNA synthesis, ribosome assembly, and protein synthesis. Molecular Systems Biology. 9:678.
- Hockenberry, A.J., and Jewett M.C. 2012. Synthetic in vitro circuits. Current Opinion in Chemical Biology. 16: 253–259.
- Harris, D.C., and Jewett M.C. 2012. Cell-free biology: exploiting the interface between synthetic biology and synthetic chemistry. Current Opinion in Biotechnology. 5:672-678.
- Carlson, E.D., Gan, R., Hodgman, C.E., and Jewett M.C. 2012. Cell-free protein synthesis: Applications come of age. Biotechnology Advances. 30:1185-1194.

Research highlights from this project

The long-term goal of this work is to enable a transformative technology platform for the production of bio-based and bio-inspired materials that have utility for the soldier using engineered translation systems (**Fig. 1**). The focus of this proposal was to, for the first time, enable the ability to engineer the core catalyst of the translation apparatus, the ribosome, *in vitro* for the production of sequence-defined polymers (SDPs). Specifically, we sought to use a newly invented method for integrated synthesis, assembly, and translation technology (termed iSAT) for the *in vitro* construction of *Escherichia coli* ribosomes in crude ribosome-free S150 extracts. iSAT technology allows for the simultaneous synthesis of ribosomal RNA (rRNA), assembly of rRNA with purified ribosomal proteins, and translation of a reporter protein as a measure of ribosome activity. Several major achievements were accomplished in this grant period, which we highlight below.

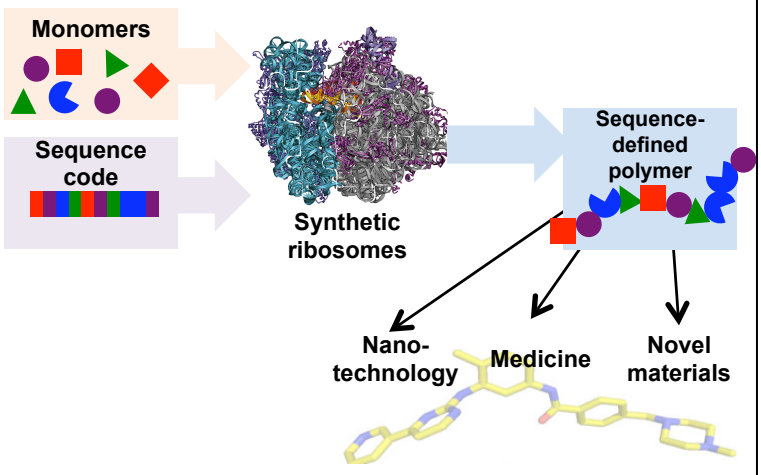

Figure. 1. Program objective: develop an enabling technology for the biosynthesis of advanced materials using engineered translation systems and synthetic ribosomes.

1. Cell-free ribosome construction. A purely *in vitro* strategy for *E. coli* ribosome construction offers a powerful technique to study and exploit engineered ribosomes. This goal has been unachievable for decades because conventional ribosome reconstitutions are non-physiological, and *E. coli* ribosomes reconstituted with *in vitro* transcribed ribosomal RNA (rRNA) are essentially nonfunctional. To overcome these limitations, we developed an integrated synthesis, assembly, and translation method (iSAT) that enables efficient one-step co-activation of rRNA transcription, assembly of transcribed rRNA with native ribosomal proteins into *E. coli* ribosomes, and synthesis of functional proteins by these ribosomes in a ribosome-free S150 extract (**Fig. 2A**). A novel feature of iSAT is that it mimics co-transcription of rRNA and ribosome assembly as it occurs *in vivo.* With support from ARO, we improved iSAT activity >1000-fold by optimizing extract preparation methods, tuning rRNA transcription, alleviating substrate limitations, and using macromolecular crowding and reducing agents (**Fig. 2B**). We have also mechanistically dissected why each improvement was observed. In one instance, we increased transcriptional efficiency through 3'-

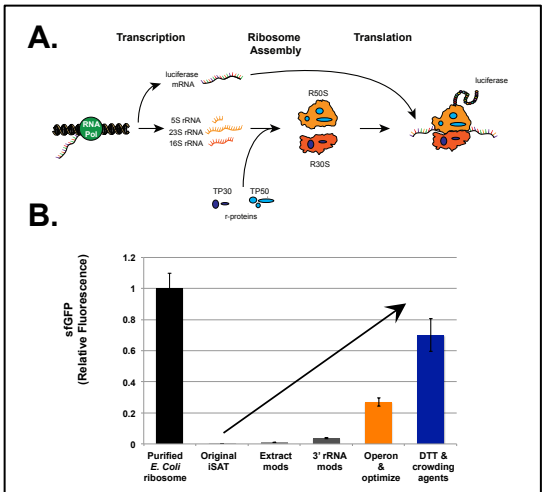

Figure 2. A. The iSAT method of constructing synthetic ribosomes. B. iSAT activity has improved >1000-fold and purified iSAT ribosomes now show ~70% the activity of purified *E. coli* 70S ribosomes.

modifications to the rRNA gene sequences, optimized plasmid and polymerase concentrations, and demonstrated the use of a T7-promoted rRNA operon for stoichiometrically balanced rRNA synthesis and native rRNA processing. Our modifications produced a 45-fold improvement in iSAT protein synthesis activity. In another study, we discovered that macromolecular crowding and reducing agents increase overall iSAT protein synthesis; the combination of 6% w/v Ficoll 400 and 2 mM DTBA yielded approximately a five-fold increase in overall iSAT protein synthesis activity. By utilizing a fluorescent RNA aptamer, fluorescent reporter proteins and ribosome sedimentation analysis, we showed that crowding agents increase iSAT yields by enhancing translation while reducing agents increase rRNA transcription and ribosome assembly. In sum, our efforts showed that iSAT ribosomes possess ~70% of the protein synthesis activity of *in vivo*-assembled *E. coli* ribosomes, which we believe to be sufficient for engineering applications.

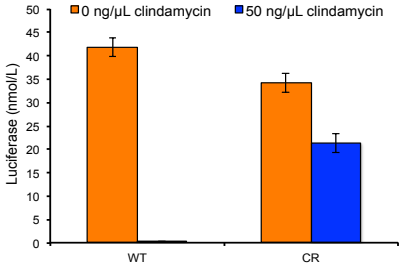

Figure 3. Demonstration that iSAT can make variant ribosomes with modified rRNA. The point mutation A2058U of the 23S rRNA gene was made to confer resistance to the antibiotic clindamycin (WT versus CR) in iSAT reactions.

Notably, the iSAT approach can easily synthesize modified ribosomes by changing the DNA input, as we have shown by introducing a 23S rRNA mutation that mediates clindamycin resistance (**Fig. 3**). Also, importantly, this platform can access mutations that are otherwise unattainable in cells (such as mutations to A2451 and C2452, which are dominant lethal in cells, and are part of the A-site on the 50S subunit). Thus, iSAT is poised to enable the construction of ribosome mutants that are dominant lethal and otherwise not observed in cells.

2. Cell-free Ribosome Synthesis and Evolution (RISE) to evolve mutant ribosomes for new function. We combined iSAT with ribosome display to create RISE, a method for selecting active, mutant ribosomes *in vitro* from a DNA library (**Fig. 4**). RISE uses mutated DNA to transcribe a library of rRNA variants that is assembled into a library of variant ribosomes in iSAT. By selectively capturing rRNA mutants capable of synthesizing a desired polymer, RISE can enrich a target 23S rRNA gene from a library >1000-fold per round of selection. We used the RISE method to explore mutations of the peptidyl transferase center of the *E. coli* ribosome and to evolve novel combinations of base mutations that convey resistance to the antibiotic clindamycin. The RISE method is a powerful approach for exploring the effects of rRNA mutations on ribosome function and to ultimately isolate ribosomal variants with altered functionalities.

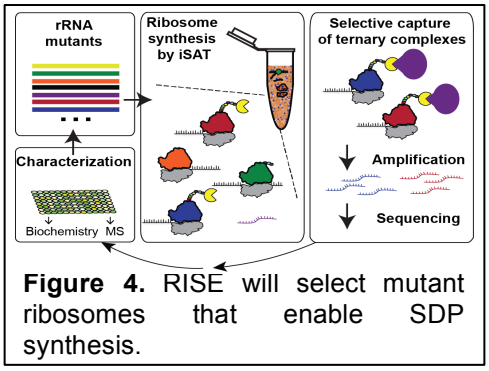

Figure 4. RISE will select mutant ribosomes that enable SDP synthesis.

Below, we show two applications of the technology. The first use of the RISE system focused on mutation of bases in the peptidyl transferase center (PTC) of the ribosome. The PTC is made of the 23S rRNA, and research has identified a region of 79 bases within the PTC that contain six post-transcriptionally-modified bases (2445, 2449, 2457, 2498, 2503, and 2504) that are required for ribosome activity. To explore the effect of mutations in this region, we created two libraries of rRNA operon plasmids, with one library, termed 6E, consisting of degenerate mutations to the six essential post-transcriptionally-modified bases of 23S rRNA. The other

library, termed 12NC, consisted of degenerate mutations to 12 bases of the critical region of the 23S rRNA PTC that are not conserved between the bacterial species *E. coli, Bacillus stearothermophilus,* and *Thermus aquaticus*: 2461, 2462, 2464, 2468, 2471, 2474, 2477, 2479, 2482, 2486, 2488, and 2489. This analysis is consistent with data from the Comparative RNA Web Site. The 6E and 12NC libraries theoretically contained 4,096 (4^6) and 1.7 x 10^7(4^{12}) members, respectively.

The RISE method was applied under normal iSAT reaction conditions to these libraries over two cycles. The initial libraries and operon pools created from recovered rRNA after each cycle were tested for activity in iSAT reactions and sent for sequencing. The activity tests show an increase in protein synthesis from rRNA operon reassembled after each RISE cycle (**Fig. 5**). Notably, the sequence traces of the initial libraries show proper degeneracy of the 6 bases of the 6E library or the 12 bases of the 12NC library, and both libraries show convergence towards the native sequence after just one RISE cycle, with nearly complete convergence after two RISE cycles (**Figs. 6 and 7**). The size of the 12NC library and rapid convergence after two RISE cycles suggests ~4,000-fold specificity for the RISE method in practice.

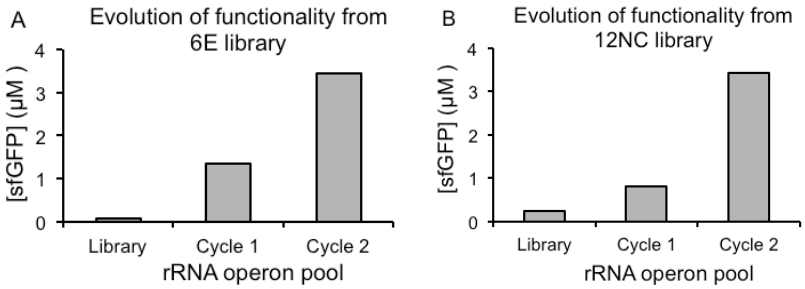

Figure 5. sfGFP production of iSAT reactions using rRNA operon pools for (A) 6E and (B) 12NC evolutions, with pools including original libraries and 1 or 2 RISE cycles. Values represent averages of two independent reactions.

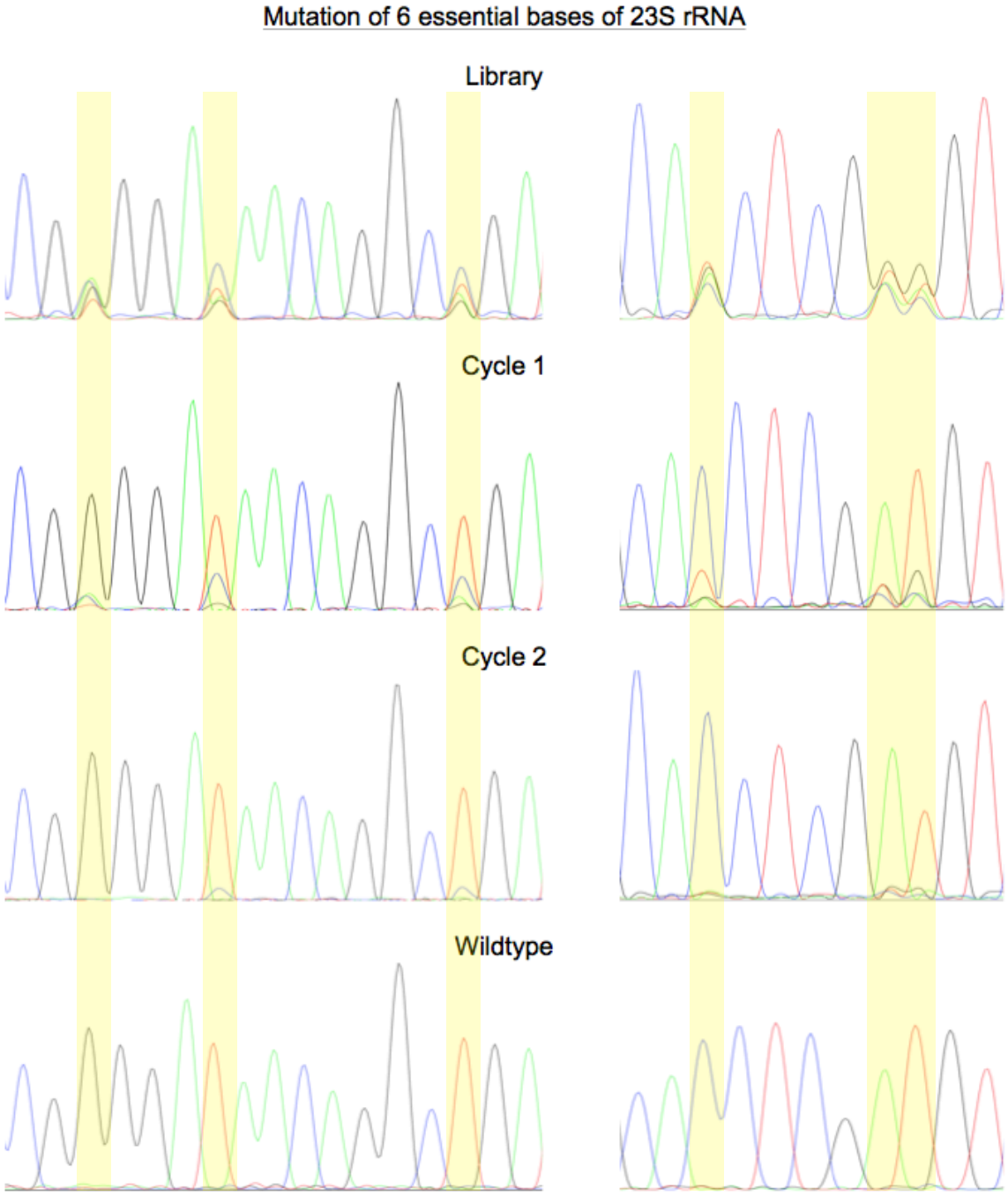

Figure 6. Progression of rRNA operon sequence composition for evolution of 6 essential 23S rRNA bases using the RISE method. Traces show bases 2443 to 2459 (left) and 2496 to 2506 (right) of 23S rRNA. Yellow is used to mark the 6 mutated bases.

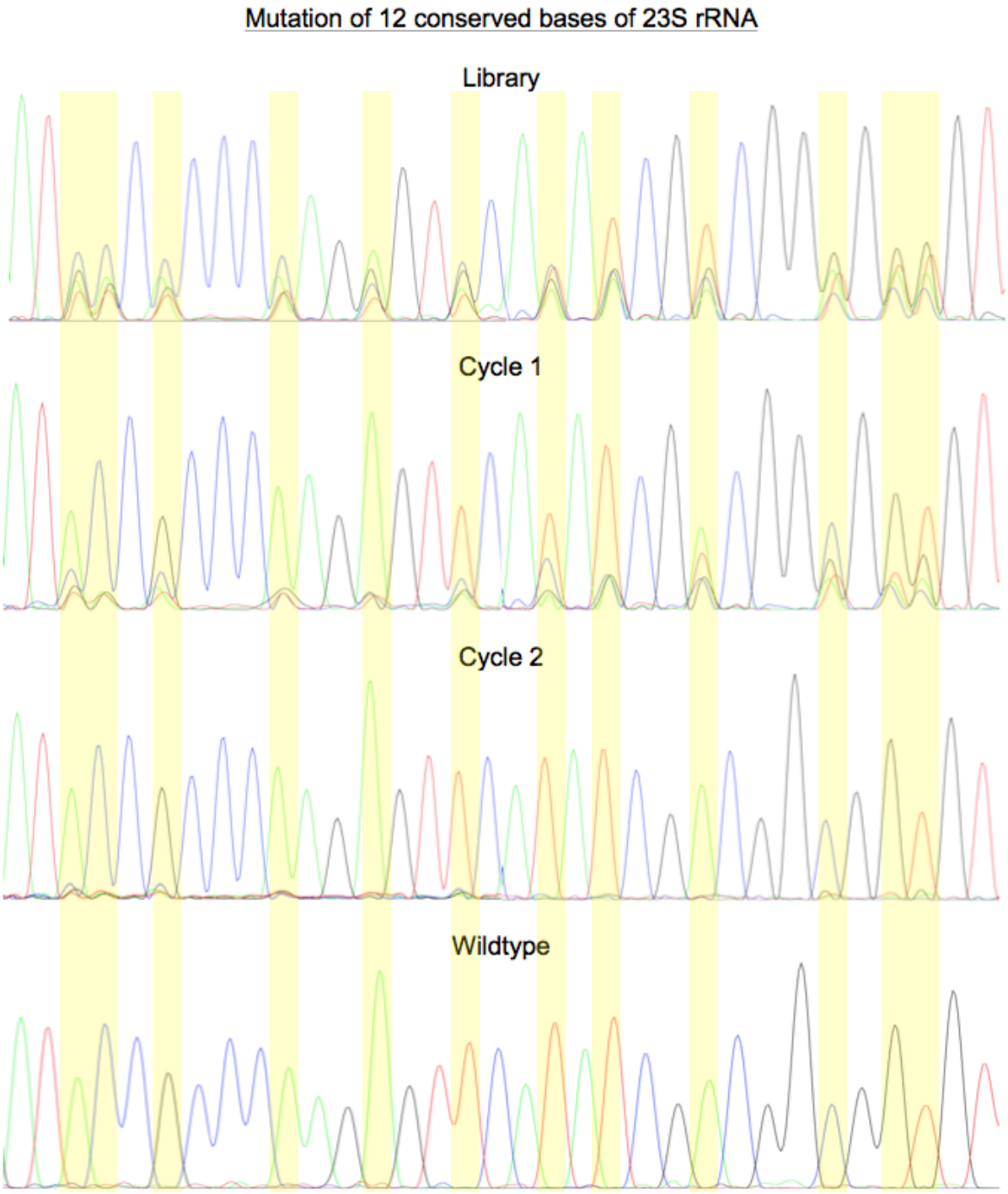

Figure 7. Progression of rRNA operon sequence composition for evolution of 12 non-conserved 23S rRNA bases using the RISE method. Traces show bases 2459 to 2491 of 23S rRNA. Yellow is used to mark the 12 mutated bases.

To demonstrate evolution of ribosomes with new functionality using the RISE method, we sought to recreate the evolution of clindamycin-resistant ribosomes from Cochella and Green (2004) in which ribosome libraries were constructed *in vivo*, purified, and used for *in vitro* ribosome display, with each cycle requiring a round of transformation, cell growth, and ribosome purification. First, we recreated the rRNA operon library by mutating 6 bases, 2057 to 2062 of 23S rRNA, which are associated with clindamycin binding. We then applied the RISE method to the clindamycin-resistance (CR) library with either 0 or 500 μM clindamycin in the iSAT reaction. After each RISE cycle, we analyzed the composition of the rRNA operon pools through sequencing. As with the 6E and 12NC library constructions, we observed that the CR library showed degeneracy at the 6 mutated bases. After three RISE cycles, the 0 μM clindamycin treatment resulted in rRNA operon pools mostly converging to the wild type sequence. However, the 500 μM clindamycin treatment showed convergence at bases 2060 and 2061, but high degeneracy at the other bases. Since the rRNA sequences from the CR library had not converged, we transformed the rRNA operon pools after 3 RISE cycles and purified and sequenced DNA encoding 23S rRNA from individual colonies (**Fig. 8**). For the 0 μM clindamycin treatment, 13 of 26 sequences contained the wild type sequence, and the other 13 sequences were each unique. However, for the 500 μM clindamycin treatment, no wild type sequences were observed from 19 sequences. Additionally, the 19 sequences resulted in 18 unique sequences, showing no convergence except for bases 2060 and 2061.

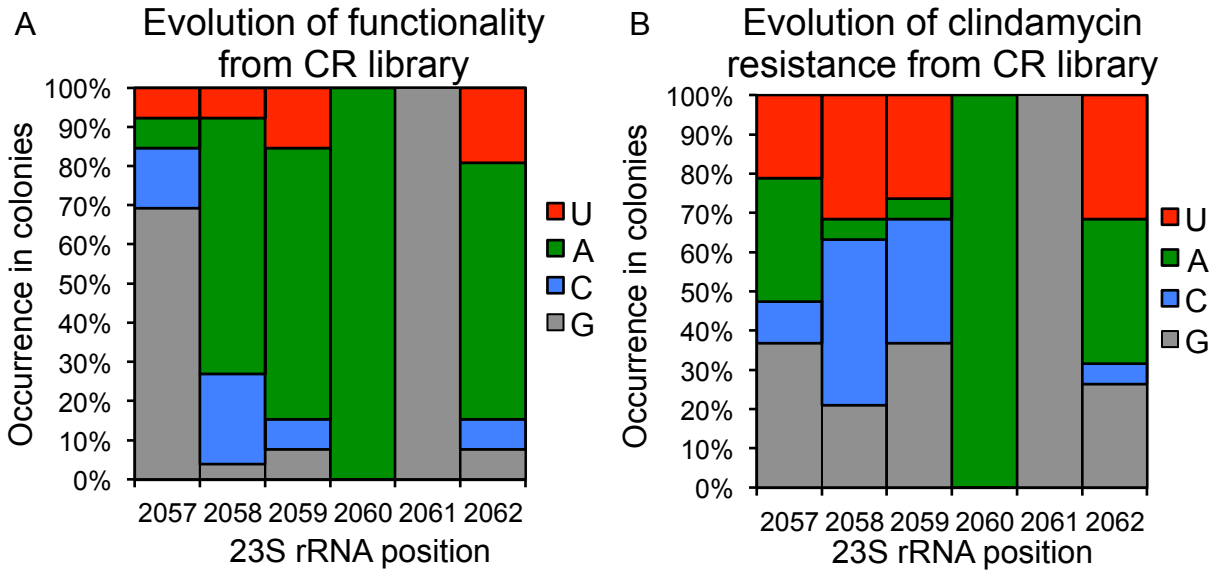

Figure 8. Distribution analysis of bases for individually sequenced rRNA operon variants recovered from evolution of clindamycin-resistance library. The CR library was evolved with either (A) 0 μM clindamycin or (B) 500 μM clindamycin. For (A), 26 variants were sequenced, and for (B), 19 variants were sequenced.

From the 500 μM clindamycin-resistance evolution, we purified plasmids from 10 colonies and tested their activity in iSAT reactions with and without clindamycin (**Fig. 9**). All 10 colonies showed significant resistance to 500 μM clindamycin, despite a variety of sequences being represented.

A

23S rRNA, 2057-2062	
Name	Sequence
WT	GAAAGA
A2058U	GUAAGA
CR1	ACCAGA
CR2	UGGAGA
CR3	CCGAGA
CR4	AUAAGU
CR5	GGCAGA
CR6	UAGAGU
CR7	GCUAGA
CR8	GUCAGG
CR9	CUGAGU
CR10	AUUAGC

B

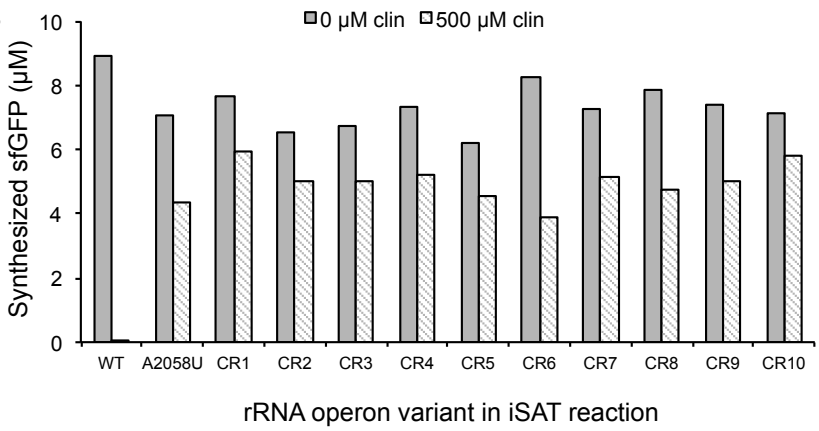

Figure 9. Sequences and activity of individual rRNA operon variants recovered from clindamycin-resistance evolution using the RISE method. (A) Table of individual variants tested in iSAT reactions. Sequences CR1 through CR10 were isolated plasmids obtained after three RISE cycles for clindamycin-resistance evolution. (B) sfGFP production of iSAT reactions using 1 nM purified plasmid from individual rRNA operon variants. Values represent averages of two independent reactions.

Development of the RISE method required merging the iSAT system for *in vitro* ribosome construction with the methods associated with ribosome display. Based on the current 15 μL scale, iSAT reactions utilize 9 x 10^9 DNA molecules of the rRNA operon plasmid. The capture of approximately 3 x 10^{11} ribosomes by the RISE method provides full coverage of DNA used in the iSAT reaction, suggesting that the amount of operon DNA used in the reactions is currently limiting RISE library size to approximately 10^9 molecules, or degeneracy of up to 15 bases.

Overall, the RISE method is an exciting new approach to exploring ribosomal mutations that removes the restrictions of cell viability and transformation efficiency encountered by *in vivo* work. We demonstrate how the RISE method can be used to evolve ribosomes of novel functionality. With this new approach, ribosomal mutants can be rapidly created and screened, providing a powerful new tool to biologists and bioengineers alike. Moving forward, we anticipate using the RISE method to probe the *E. coli* ribosome for mutatable regions, to determine which ribosomal structures can be deleted while preserving ribosome functionality, and to introduce mutations that favor unnatural amino acid incorporation. We hope to publish the RISE story in the coming year.

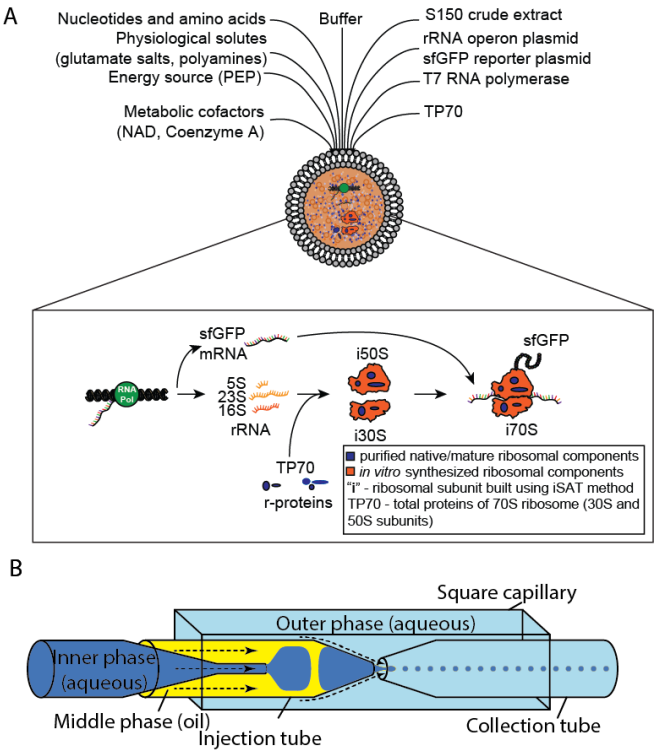

Figure 10. (A) Illustration of the iSAT system and its components. (B) Schematic of the microfluidic capillary device to generate ultra-thin double emulsions encapsulating iSAT solution.

3. Compartmentalized cell-free ribosome construction. In a parallel route to ribosome display, we sought to explore the ability to encapsulate the cell-free ribosome construction platform (**Fig. 10**). In a paper published in *Chemical Communications*, we demonstrated the ability to build functionally active ribosomes in giant liposomes. The liposomes were prepared using double emulsion template, and compartmentalized *in vitro* protein synthesis was analyzed using spinning disk confocal microscopy. To our knowledge, this is the first time that a cell-free transcription and translation system where the DNA molecule encoding the formation of ribosomes has been encapsulated in a model cell-like compartment, *i.e.* liposome, and thus represents a major step towards the construction of a minimal cell. In addition, it represents an important step towards *in vitro* evolution of ribosomes in emulsions.

4. iSAT generates functional ribosomes with mutations in the peptidyl transferase center (PTC). A core hypothesis of our long-term vision is that mutations in the PTC of the ribosome can promote alternative poly-condensation reactions to enable novel sequence defined polymers. Testing this requires the ability to rapidly build mutant ribosomes, which we demonstrate here. Using iSAT, we assembled mutant ribosomes possessing single base substitutions at 23S rRNA nucleotides A2451, C2452, U2506, U2585, and A2602. These nucleotides are highly conserved in the PTC and their substitution *in vivo* can confer a dominant lethal phenotype (*e.g.,* A2451C/U). **Figure 11** illustrates full-length protein synthesis kinetics of iSAT-assembled WT and mutant ribosomes. This attribute is critical, as rRNA mutations required for alternative catalysis by the PTC could disfavor peptide bond formation. Beyond the nucleotides shown, we have additionally constructed 125 single point ribosome mutants and are assessing activity and fidelity from these constructs. In the future, our work

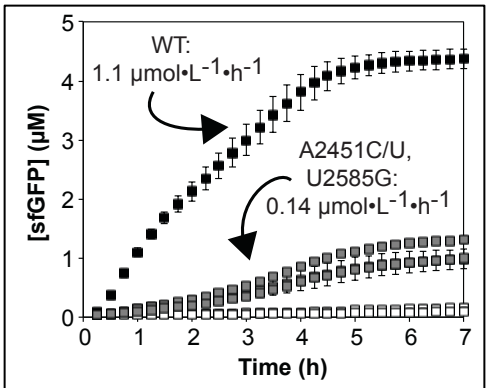

Figure 11. iSAT supports the synthesis of mutant ribosomes. Average translation rates of WT iSAT ribosomes and those (A2451C/U,U2585G) whose mutations would be lethal in cells.

to catalog mutant ribosomes will chiefly set the stage for future efforts to use backbone modified amino acid analogs or different chemistries altogether.